

## MOLECULAR CLONING, EXPRESSION AND FUNCTIONAL INTERACTION OF p48 SUBUNIT OF CHICKEN CHROMATIN ASSEMBLY FACTOR 1 WITH HISTONE DEACETYLASE 2 AND HISTONE ACETYLTRANSFERASE 1

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### ABSTRACT

We cloned and sequenced cDNA encoding p48 subunit of the chicken CAF-1, chCAF-1p48, and histone acetyltransferase-1, chHAT-1 from chicken DT40 cell lines. We showed that the p48 subunit of CAF-1 tightly binds to two regions of chicken histone deacetylase 2, chHDAC-2, located between amino acid residues 82-180 and 245-314, respectively. We also established that two N-terminal, two C-terminal, or one N-terminal and one C-terminal WD repeat motif of chCAF-1p48 are required for this interaction. The GST pulldown assay, involving truncated and missense mutants of chCAF-1p48, revealed not only that a region containing the seventh WD dipeptide motif of chCAF-1p48, comprising amino acids 376-405, binds to chHAT-1 in vitro, but also that mutation of the motif has no influence on the in vitro interaction. We also established that the region, which is located between amino acids 380-408 of chHAT-1 and contains a leucine zipper motif, is required for its in vitro interaction with chCAF-1p48. Mutation on each of four Leu residues in the leucine zipper motif of chHAT-1 causes the disappearance of the interaction with chCAF-1p48. These results should be useful information for understanding the participation of chCAF-1p48 protein as histones chaperone in DNA-utilizing processes, such as replication, recombination, repair and gene expression in DT40 chicken B cell.

**Keywords:** Chromatin assembly factor-1; histone deacetylase 2; histone acetyltransferase 1; polymerase chain reaction; polyacrylamide gel electrophoresis

### ABSTRAK

Telah dilakukan penelitian yang bertujuan mengkloning dan menentukan urutan cDNA dari gen yang menyandi subunit p48 dari CAF-1 (CAF-1p48), histone deasetilase-2 (HDAC-2), dan histone asetil transferase-1 (HAT-1) pada sel ayam DT40. Hasil penelitian menunjukkan bahwa subunit p48 dari CAF-1 dapat berinteraksi dengan histone deasetilase-2 pada dua lokasi yaitu masing-masing antara residu asam amino 82-180 dan 245-314. Sebaliknya ada dua lokasi pada N-terminal, dua lokasi pada C-terminal atau masing-masing satu lokasi pada N-terminal dan satu lokasi pada C-terminal dari CAF-1p48 diperlukan untuk berinteraksi dengan histone deasetilase-2. Dengan teknik GST pulldown assay, tidak hanya menemukan bahwa lokasi ke tujuh dari motif WD dipeptida antara residu asam amino 376-405 dari CAF-1p48 dapat berinteraksi dengan HAT-1, tetapi mutasi pada motif tersebut tidak mempengaruhi interaksi kedua jenis protein. Pada residu asam amino 380-408 dari HAT-1 terdapat leucine zipper motif yang esensial untuk berinteraksi dengan CAF-1p48. Hasil penelitian ini dapat memberikan informasi untuk memahami peranan protein CAF-1p48 dalam berbagai aktivitas molekul DNA seperti proses replikasi, rekombinasi, dan ekspresi gen dalam sel ayam DT40.

**Kata Kunci:** Chromatin assembly factor-1; histone deasetilase-2; histone asetiltransferase 1; reaksi polimerisasi berantai; elektroforesis gel poliakrilamida

### INTRODUCTION

In the nucleus of the eukaryotes cells, DNA is packaged into a nucleoprotein structure known as chromatin. The basic repeating unit of chromatin, the nucleosome, consists of approximately two turns of DNA wrapped around an octamer of core histone proteins [1].

Understanding of the mechanisms of chromatin assembly and alterations in the chromatin structure in eukaryotes is a fundamental goal, because they have been thought to be predominantly involved in DNA-utilizing processes, such as replication, recombination, repair and gene expression.

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Stable homeostasis of a living cell is a direct result of coordination of a large number of molecular interaction events. An approach to study these protein functions is to clarify a mechanism for the interaction with each other. Classical activators contain distinct activation domain and function through interaction either directly or indirectly of nuclear proteins with components of the basal transcription machinery. One of the repression activities of transcription of gene is dependent on a conserved and modified motif of nuclear protein; such as acetylation and deacetylation of the histones is catalyzed by histone acetyltransferase and histone deacetylase. The level of acetylation is related to transcriptional activity, and the acetylation induces an open conformation that allows the transcription machinery access to promoters [2].

Transcriptional repression by nuclear receptors is mediated by a complex containing SMRT or N-CoR, mSin3 and HDACs. Mammalian HDAC-1 is recruited by the retinoblastoma protein to repress transcription [3-4]. Thus, repressor complexes, like the chromatin remodeling complex, contain HDACs that deacetylate nucleosomal core histones, producing alterations of the chromatin structure that block transcription. Moreover, interestingly, these repressor complexes contain the p48 subunit, which is also contained in the chromatin assembly factor-1 (CAF-1) involved in chromatin assembly in eukaryotes, together with two other subunits, p60 and p150 [5-6]. The p48 subunit, CAF-1p48, with seven WD repeat motifs, is a member of the WD protein family [7-8]. CAF-1p48 is essential for the viability of DT40 cells (and probably most vertebrate cells) [9-10], although the depletion of CAF-1 is not lethal for yeast [11].

p48 subunit of CAF-1 is associated with histone H4 in the absence of other CAF-1 subunits, p60 and p150 [12], suggesting that it links predeposited histones and various chromatin-related complexes. CAF-1 is involved in DNA replication/repair-dependent nucleosome assembly [13]. The proposed mechanism is as follows. First, CAF-1 is recruited onto replicated DNA, or DNA containing single-strand breaks, through PCNA in an ATP-dependent manner [14]. The recruitment of CAF-1 to DNA damage sites is mediated by Werner syndrome protein WRN, which belongs to the RecQ family of DNA helicase [15]. Second, the CAF-1-mediated nucleosome assembly reaction proceeds in two steps. CAF-1 first deposits histones H3 and H4 onto replicated DNA, and histone H2A-H2B dimers subsequently bind to the histone (H3-H4)<sub>2</sub> tetramer [16]. In the first step, CAF-1 selectively binds to histone H3.1 and then facilitates DNA replication/repair-dependent nucleosome assembly reaction coordinately with ASF-1 in vitro [17-18]. Third, CAF-1 forms a complex with methyl CpG binding protein MBD1 and H3-K9 methyltransferase SETDB1 in the S

phase [19]. Fourth, p150 colocalizes with PCNA and HP1, which recognizes methylated H3-K9, to replicated heterochromatin regions [20]. Although a functional effect on heterochromatin at telomeres is observed in the CAF-1 mutant, there is no structural effect on telomeric heterochromatin [21], suggesting that CAF-1 possesses an unknown function that regulates heterochromatin-specific gene expression.

As a first step to clarify the mechanisms of DNA-utilizing processes, we have studied the interaction of the p48 subunit of the chicken chromatin assembly factor-1 with histone-modifying enzymes especially histone deacetylase 2 (HDAC-2) and histone acetyltransferase 1 (HAT-1) which exist in multiple forms in various organisms, including chicken, mouse and human.

## EXPERIMENTAL SECTION

### Materials

pBluescript II SK(-) and pCite4a(-) were purchased from Stratagene and Novagen, respectively. pGEX-2TK plasmid, glutathione-Agarose beads, radioisotope [<sup>35</sup>S]-Met, and fluorographic reagent were purchased from Amersham Pharmacia Biotech. A single Tube Protein™ System 3 were purchased from Novagen. The GAL 4-based system and yeast *S. cerevisiae* MaV203 strain were obtained from Gibco BRL.

### Instrumentation

Instruments used in this work are DNA sequencing (Perkin-Elmer) Applied Biosystems Division, model 310 for sequencing of cDNA and point mutation plasmid mutants. PCR machine was used for gene cloning and preparation of point mutation plasmid mutants. Ultrasonication apparatus was used for cell lysis. Electrophoresis apparatus and Immunoprecipitation Apparatus were used for protein-protein interaction analysis. Autoclave, Clean Bench, Shimadzu UV/Vis Spectrometer and shaker incubator were used for bacterial preparation.

### Procedure

#### Cloning and sequencing of cDNA encoding chCAF-1p48

The cloning and sequencing of cDNA encoding chCAF-1p48 were performed by standard procedures essentially as described previously [22-23]. The entire nucleotide sequences of both strands of the largest cDNA insert were sequenced by the dye terminator method (Applied Biosystems Division, Perkin-Elmer).

### Expression and purification of GST fusion proteins

*E. coli* BL-21 cells were transformed with pGEX-2TKchCAF-1p48 and pGEX-2TKchHDAC-2, respectively, harboring the full-length chCAF-1p48 and chHDAC-2 cDNAs and grown to  $A_{600\text{ nm}} = 0.2$  in 400 mL of LB medium supplemented with 200  $\mu\text{g/mL}$  ampicillin. Upon induction with 50  $\mu\text{M}$  isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) overnight at 20  $^{\circ}\text{C}$ , the cells were collected by centrifugation and suspended in 10 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 0.1% (v/v) phenylbenzoylsulfonyl fluoride and 1  $\mu\text{g/mL}$  lysozyme, in liquid  $\text{N}_2$  for 2 min, followed by ultrasonication for 3 min. Continuously, the GST fusion proteins were purification with glutathione-agarose beads essentially as described [22-23].

### Immunoprecipitation experiment and GST pulldown affinity assay

The *in vitro* binding assays were performed, using immunoprecipitation experiment and GST pulldown affinity assay essentially as described [22-24], in 200  $\mu\text{L}$  of the bead-binding buffer.

### Yeast two-hybrid assay

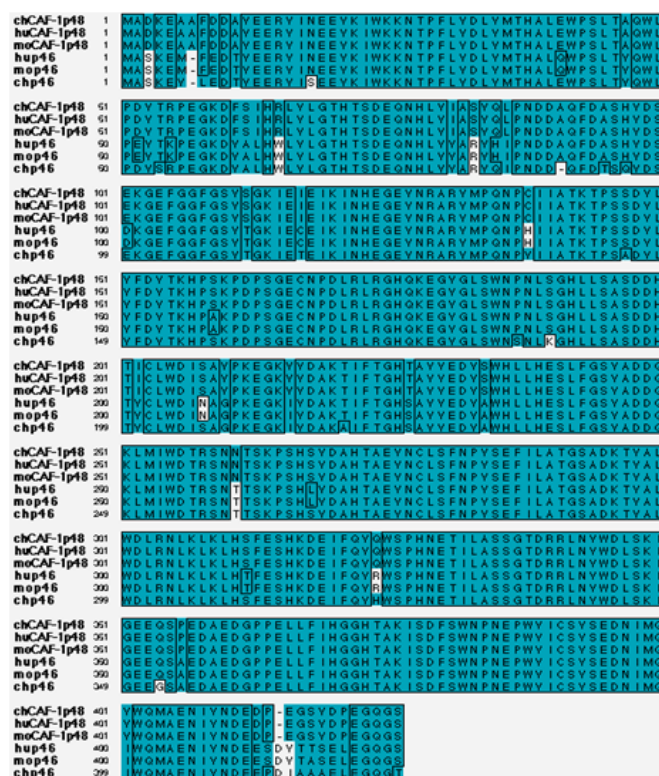
Yeast two-hybrid studies were carried out using the GAL 4-based system and yeast *S. cerevisiae* MaV203 strain as obtained from Gibco BRL product, were performed as recommended by supplier (Gibco BRL), and essentially as described previously [23].

## RESULT AND DISCUSSION

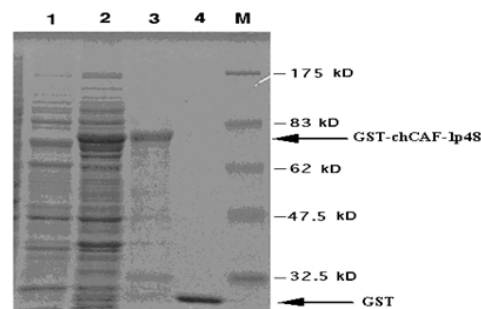
### Cloning and Expression of chicken CAF-1p48 in *E. coli* strain BL-21

We cloned and sequenced cDNAs encoding the p48 subunit of chicken CAF-1, chCAF-1p48 [22] and chicken histone acetyltransferase, chHAT-1 [23]. chCAF-1p48 consists of 425 amino acids including a putative Met, possesses seven WD repeat motifs, and is completely identical with the human and mouse CAF-1p48s in amino acid sequence (Fig. 1). chHAT-1 comprises 408 amino acids, exhibits 80.4% identity to the human homolog, and possesses a typical leucine zipper motif within its C-terminal region at position 390-408 [23,25].

To construct a chimeric plasmid, pGEX-2TKchCAF-1p48, expressing the GST-chCAF-1p48 fusion protein, chCAF-1p48 cDNA was subcloned into the pGEX-2TK plasmid in frame. GST fusion proteins were synthesized in *E. coli*, extracted, and purified essentially as described above. As shown in Fig. 2, the electrophoretic patterns on SDS-PAGE of whole cell lysates before and after the induction with 50  $\mu\text{M}$  IPTG

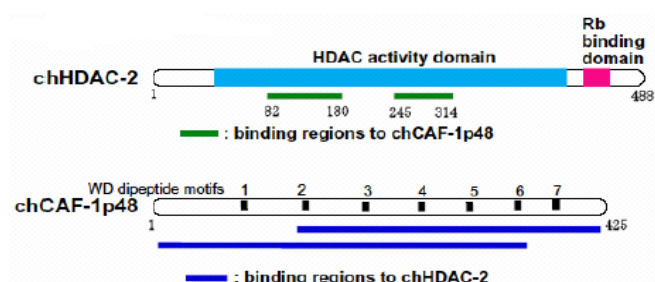


**Fig 1.** Comparison of aligned amino acid sequences of p48 subunit of chromatin assembly factor-1s. The proteins used are denoted by appropriate abbreviations, i.e. chCAF-1p48 is the abbreviation for chicken chromatin assembly factor-1 p48 subunit

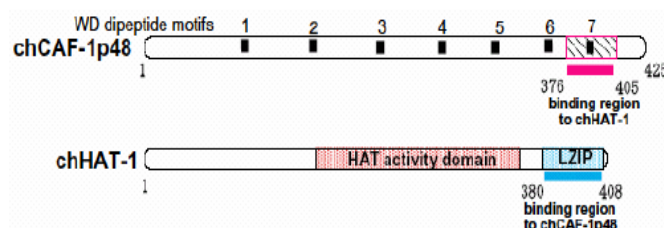


**Fig 2.** SDS gel electrophoretic patterns of GST-chCAF-1p48 fusion protein-containing fractions at different purification steps. Lane 1, whole cell lysate of strain BL-21 cells containing the pGEX-2TKchCAF-1p48 plasmid without induction by IPTG; lane 2, the lysate with induction by IPTG; lane 3, the fraction purified with glutathione-agarose beads; lane 4, GST; lane M, molecular mass standards

revealed that GST-chCAF-1p48 fusion proteins of approximately 74 kD were dramatically accumulated in *E. coli* strain BL-21 cells containing the pGEX-2TKchCAF-1p48 plasmid. In addition, the GST-chCAF-1p48 fusion proteins were purified to more than 95%



**Fig 3.** Essential regions for the interaction of chCAF-1p48 and chHDAC-2. The proteins used are denoted by appropriate abbreviations, i.e. Rb is the abbreviation for Retinoblastoma protein



**Fig 4.** Essential regions for the interaction of chCAF-1p48 and chHAT-1. The protein regions used are denoted by appropriate abbreviations, i.e. LZIP is the abbreviation for leucine zipper motif

		Binding to chCAF-1p48		
		<i>in vitro</i>	<i>in vivo</i>	
		GST pulldown	IP	two-hybrid
HAT-1(Wild-type)	-----LTNQLNQIMMQLBQLBESFQQLVSDYRRLERLAQA	+	+	+
HAT-1(L380A)	.....A.....L.....L.....L.....	-	-	-
HAT-1(L387A)	.....L.....A.....L.....L.....	-	-	-
HAT-1(L394A)	.....L.....L.....A.....L.....	-	-	-
HAT-1(L402A)	.....L.....L.....L.....A.....	-	-	-

**Fig 5.** Summary of the necessity of the leucine zipper motif of chHAT-1 for the *in vitro* and *in vivo* interaction with chCAF-1p48. The experimental methods are denoted by appropriate abbreviations, i.e. IP is the abbreviation for immunoprecipitation

homogeneity, using glutathione-agarose beads (see lane 3 in Fig. 2).

**At least two N-terminal WD repeats and two C-terminal WD repeats of chCAF-1p48, and two regions of chHDAC-2 are necessary for their interaction**

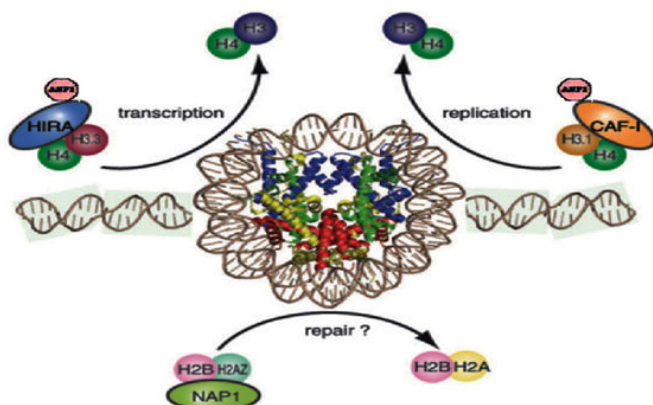
An immunoprecipitation experiment followed by Western blotting showed that chCAF-1p48 interacts with chHDACs *in vivo* [22]. A GST pulldown affinity assay revealed the *in vitro* interaction of chCAF-1p48 with chHDAC-1, -2, and -3. We established that the p48 subunit binds to two regions of chHDAC-2, comprising amino acids 82-180 and 245-314, respectively (Fig. 3). Using deletion mutants of the respective regions, we also showed that two N-terminal, two C-terminal, or one N-terminal and one C-terminal WD repeat motif of chCAF-1p48 are required for this interaction. These results suggest that chCAF-1p48 is involved in many aspects of DNA-utilizing processes, through alterations in the chromatin structure based on the modification of core histones with acetyl groups.

**The leucine zipper motif of chHAT-1 and C-terminal region of chCAF-1p48 are necessary for the interaction of the two proteins**

By GST pulldown assay, involving truncated and missense mutants of chCAF-1p48, we established that not only a region containing the seventh WD dipeptide motif of chCAF-p48, comprising amino acids 376-405, binds to chHAT-1 *in vitro*, but also that mutation of the motif has no influence on the *in vitro* interaction (Fig. 4). A GST pulldown assay, involving truncated and missense mutants of chHAT-1, revealed not only that a region comprising amino acids 380-408 of chHAT-1 (containing the leucine zipper motif) is required for its *in vitro* interaction with chCAF-1p48, but also that mutation of each of the four Leu residues in the leucine zipper motif prevents the interaction of the two proteins.

A yeast two-hybrid assay revealed not only that chHAT-1 binds to chCAF-1p48 *in vivo*, but also that all four Leu residues within the leucine zipper motif of chHAT-1 are necessary for its interaction with chCAF-1p48. These results indicate that the proper leucine





**Fig 6.** A role of chromatin assembly factor-1 (CAF-1) and HIRA protein as histone chaperons on DNA transcription, replication, and repair process for the maintenance of the cell viability

zipper motif of chHAT-1 is essential for the interaction with chCAF-1p48, whereas the propeller structure of chCAF-1p48 expected to be a platform for protein-protein interactions may not be necessary for this interaction.

Taken together, as summarized in Fig. 5, these results indicated that the proper structure of the leucine zipper motif is essential for the *in vitro* and *in vivo* interaction of chHAT-1 with chCAF-1p48. As described [26], the human HAT-1 could not associate with the human p48 subunit *in vivo*. The reason for the discrepancy between our results as to the interaction of chHAT-1 with chCAF-p48 and those for the interaction of the human HAT-1 with the p48 subunit is likely that the two Leu residues at positions 380 and 402 within the leucine zipper motif of chHAT-1 are substituted by Ile residues in the corresponding region of the human HAT-1.

In this work, we cloned and sequenced cDNA encoding the small subunit of the chicken CAF-1, chCAF-1p48. It consists of 425 amino acid residues including a putative initiation Met, possesses seven WD repeat motifs, which was completely identical with the human and mouse CAF-1p48s. To systematically assess the role of chCAF-1p48, we also cloned cDNA encoding the chicken cytoplasmic histone acetyltransferase-1, chHAT-1, comprising 408 amino acids including a putative initiation Met. It exhibits 80.4% identity to the human homolog and possesses a typical leucine zipper motif. The immunoprecipitation experiment followed by Western blotting revealed that chCAF-1p48 interacts with chicken histone deacetylases (chHDAC-1 and -2) *in vivo*. The GST pulldown affinity assay revealed the *in vitro* interaction of chCAF-1p48 with chHDAC-1, -2 and -3. We showed that the p48 subunit of CAF-1 tightly binds to two regions of chHDAC-2, located between amino acid residues at position 82-

180 and 245-314, respectively. We also established that two N-terminal, two C-terminal, or one N-terminal and one C-terminal WD repeat motif of chCAF-1p48 are required for this interaction, using deletion mutants of the respective regions. The GST pulldown assay, involving truncated and missense mutants of chCAF-1p48, a member of the WD protein family, revealed not only that a region containing the seventh WD dipeptide motif of chCAF-1p48, comprising amino acids 376-405, binds to chHAT-1 *in vitro*, but also that mutation of the motif has no influence on the *in vitro* interaction. The GST pulldown assay, involving truncated and missense chHAT-1 mutants, established not only that a region comprising amino acids 380-408 of chHAT-1 and containing the leucine zipper motif is required for its *in vitro* interaction with chCAF-1p48, but also that mutation of each of four Leu residues in the leucine zipper motif prevents the *in vitro* interaction. The yeast two-hybrid assay revealed not only that chHAT-1 binds to chCAF-1p48 *in vivo*, but also that all four Leu residues within the leucine zipper motif of chHAT-1 are necessary for its *in vivo* interaction with chCAF-1p48.

In addition, CAF-1 localizes near replication forks and transfers epigenetic information encoded on parental nucleosomes to daughter nucleosomes. CAF-1 is associated with histone H4 acetylated at some N-terminal tail residues, namely lysines 5, 8, or 12, acetylation patterns that are characteristic of newly synthesized histones. The N-terminal tail regions of histones H3 and H4, however, are not required for interaction with CAF-1 or for CAF-1-mediated nucleosome assembly [27]. Since these acetylations, which appear to function as markers of newly synthesized histones, can be recognized by other chromatin factors, the discrimination between newly synthesized and old histone proteins might be utilized in some nuclear events. The acetylation of the newly synthesized histones appears to be removed in daughter chromatin, and the parental histone modifications are then recovered by an unknown mechanism. Studies of the functional roles of CAF-1 in nucleosome replication will be an important issue for elucidating the mechanisms underlying the inheritance of epigenetic information.

As illustrated in Fig. 6, during DNA transcription, replication, and repair, CAF-1 containing p48 subunit assembles new nucleosomes through a two-step reaction. Coupled to DNA replication, as the first step, histones H3 and H4 are deposited through a reaction that is preferentially dependent upon CAF-1, but histones H2A and H2B are added later to this immature nucleosome precursor, even in the absence of CAF-1. These results indicate that CAF-1 interacts preferentially with H3 and H4, whereas NAP-1 (Nucleosome Assembly Protein 1) binds to H2A and

H2B. On the other hand, ASF-1 was found to be histone chaperones via interactions with CAF-1p60 and HIRA proteins that modulate the formation of nucleosomes. Thus, these CAF-1-participating interactions with HIRA and HDAC-1 (and probably other CAF-1-carrying complexes including ASF-1) should be involved directly or indirectly in chromatin assembly or maintenance and alterations of chromatin structure, involving gene replication and transcription to the maintenance and support of the cell viability. Our preliminary analysis of the HIRA-deficient conditional DT40 mutant and the essential role of HIRA protein in transcriptional regulation [9-10], together with the ASF-1 conditional mutant cells [28-29] and Facilitates Chromatin Transcription (FACT), another histone chaperone protein [30], will be of powerful tools for understanding the role of CAF-1 and the other histones chaperones in relation to regulations of DNA transcription, replication, and repair to the maintenance and support of the cell viability and growth in DT40 chicken B cell line.

## CONCLUSION

In conclusion, the results of this study suggest that chCAF-1p48 is involved in many aspects of DNA-utilizing processes, through alterations in the chromatin structure based on both the acetylation and deacetylation of core histones, and the proper leucine zipper motif of chHAT-1 is essential for its interaction with chCAF-1p48, whereas the propeller structure of chCAF-1p48 expected to be a platform for protein-protein interactions may not be necessary for the interaction of chHAT-1 with chCAF-1p48. I believe our findings will contribute to a major break-through in future studies on the specific, individual roles of CAF-1, HAT-1 and HDACs involved in numerous DNA-utilizing processes, through the formation and/or maintenance of the chromatin structure in higher eukaryotes, including replication, recombination, repair and gene expression.

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